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Expression of Tetanus Toxin Subfragments In Vitro and Characterization of Epitopes

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To define epitopes of tetanus toxin, we compared four different in vitro systems in terms of their ability to produce tetanus toxin-specific subfragments from cloned DNA. A transcription-translation system developed from a nontoxigenic strain of *Clostridium tetani* was found to yield predominantly full-sized peptides. Such peptides were used to map six different epitopes for eight monoclonal antibodies. The toxin-neutralizing properties of the antibodies were determined in an in vitro assay, based on the toxin-mediated inhibition of norepinephrine release from rat brain particles. Two monoclonal antibodies recognizing epitopes within the regions Ser-744 to Ser-864 and Ile-1224 to Asp-1315 could neutralize the toxin. A third nonneutralizing antibody was shown to recognize the synthetic peptide Phe-947 to Glu-967 derived from the tetanus toxin sequence. This peptide contains a human T-cell epitope.

Tetanus toxin is a highly potent neurotoxin produced by the anaerobic bacterium *Clostridium tetani*. The toxin blocks release of inhibitory transmitter substances from central synapses (21) after a process that involves binding to ganglioside receptors at the nerve termini, receptor-mediated endocytosis, intra-axonal transport, and transsynaptic transfer (13, 25).

The structural gene for the toxin is located on a 75-kilobase (kb) plasmid (8, 11, 16) and has been sequenced recently (8, 9). The toxin (M_r 150,700) is synthesized as a single-chain polypeptide. Upon release from the bacterium, it is processed by endogenous proteases into two subunits, designated A and BC, which are held together by a single disulfide bond (4, 8). Proteolytic cleavage is paralleled by an increase in toxicity (3, 35), and the A-fragment is translocated into the cytoplasm to block transmitter release (2).

Efficient resistance to tetanus is induced by vaccination with formaldehyde-treated toxin or toxin subfragments (10, 15, 34). In this study we searched for an in vitro system that allows the characterization of toxin-neutralizing antibodies. For this purpose, we first compared different cell-free systems for their capacity to produce nontoxic peptides from cloned toxin-specific DNA. We show that a transcription-translation system established from a nontoxigenic strain of *C. tetani* was superior to other systems in furnishing full-sized peptides. Second, we mapped epitopes for monoclonal antibodies originally raised against tetanus toxin or toxoid with recombinant polypeptides. Third, two antibodies, binding to different epitopes within the C-terminal part of the BC-fragment, blocked the toxin-specific effects on the release of norepinephrine from rat brain homogenates in vitro. A third antibody which did not show the toxin-neutralizing effect was inhibited by a synthetic peptide carrying a human T-cell epitope.

MATERIALS AND METHODS

Bacteria and plasmids. *C. tetani* EK11, a nontoxigenic variant of E88 (strain Massachusetts), was kindly provided by Dr. Engelhardt (Behringwerke, Marburg, Federal Republic of Germany [FRG]). *Escherichia coli* JM101 (23) and HB101 (6) were used for purification of pUC12 and pUC13, pSP64 and pSP65 (22), or pGEM1 and pGEM2 plasmid vectors (Promega Biotech, Madison, Wis.). The pEx31a, pEx31b, and pEx31c vectors (32) carrying a polylinker in three reading frames within the MS2 polymerase gene were kindly provided by E. Beck (Zentrum für Molekularbiologie, Heidelberg, FRG).

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, Klenow polymerase, SP6 and T7 RNA polymerases, RNasin, and nucleotides were purchased from Boehringer. L-[^{35}S]methionine (>800 Ci/mmol), [^{3}H]norepinephrine (43.7 Ci/mmol), and Amplify were from Amersham Buchler (Braunschweig, FRG); diethylpyrocarbonate was from Sigma Chemical Co. (Deisenhofen, FRG); and protein A-Sepharose was from Pharmacia (Freiburg, FRG).

Cloning of DNA. Cloning of defined tetanus toxin-specific DNA fragments (Table 1) was performed under L2B1 containment following standard protocols (18). For nucleotide numbers defining the positions within the tetanus toxin gene, see Eisel et al. (8). The vectors pEJ5 and pEJ97, described previously (8), contain the toxin promoter region within 548 and 322 5′ noncoding nucleotides, respectively, placed upstream from the *tet5* and *tet97* sequences listed in Table 1.

In vitro synthesis of capped mRNA. The details of in vitro transcription have been described previously (19). The characteristics of the transcripts and of the corresponding peptides are summarized in Table 1.

In vitro translation of toxin-specific mRNA. Nuclease-treated wheat germ extract was purchased from Bethesda Research Laboratories (Neu-Isenburg, FRG) and optimized with *tet5*-specific RNA at 25°C. Optimal results were obtained with 1.0 μg of RNA in a 15-μl assay mix. Nuclease-treated rabbit reticulocyte lysate was obtained from Amersham. Translation reactions were performed according to the manufacturer's protocol. A 15-μl assay mix contained 1.0 μg of RNA and 10 μCi of [^{35}S]methionine.

Expression in a cell-free system from *E. coli*. A DNA-directed nuclease-treated S30 extract from *E. coli* was purchased from Amersham and optimized by the method of Pratt (27) with pAT153 DNA as a control. A 15-μl assay mix

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TABLE 1. Plasmid vectors for the synthesis of tetanus toxin-specific polypeptides

Designation	Parental vector[a]	Tetanus toxin-specific sequences[b]	Length of transcript[c] (nt)	Size of polypeptide[d] (Da)
pSP64tet5	pSP64 (*Hin*cII-*Bam*HI)	−2 to 1215 (*Sna*BI-*Bgl*II)	1,259	46,935
pSP64tet97	pSP64 (*Hin*cII-*Eco*RI)	−2 to 1604 (*Sna*BI-*Eco*RI)	1,630	61,806
pGEMtet6	pGEM1 (*Hin*dIII-*Hin*cII)	1215 to 2227 (*Bgl*II-*Bgl*II)[e]	1,076	38,382
pGEMtet266	pGEM2 (*Eco*RI-*Hin*dIII)	2002 to 2774 (*Hpa*II-*Hpa*II)[f]	820	28,843
pGEMtet9	pGEM1 (*Hin*cII-*Eco*RI)	2401 to 2774 (*Xba*I-*Eco*RI)[g]	1,292	47,392
pEJ4	pEJ97 (*Sna*BI-*Eco*RI)	3155 to 3673 (*Sca*I-*Eco*RI)		19,657
pEJ5	pUC12 (*Bam*HI)	−548 to 1215 (*Bgl*II)		46,935
pEJ97	pUC12 (*Hin*dIII-*Eco*RI)	−322 to 1604 (*Hin*dIII-*Eco*RI)		61,806
pEJ6	pUC12 (*Bam*HI)	1215 to 2227 (*Bgl*II-*Bgl*II)		38,382
pExtet51	pEx31b (31) (*Bam*HI)[g]	−2 to 273 (*Sna*BI-*Nco*I)[g]		23,021
pExtet52	pEx31b (*Bam*HI-*Bgl*II)[g]	270 to 1218 (*Nco*I-*Bgl*II)[g]		47,225
pExtet53	pEx31b (*Bam*HI)[g]	−2 to 735 (*Sna*BI-*Bsm*I)[h]		40,021
pExtet31	pEx31a (*Bgl*II-*Bam*HI)[g]	2228 to 3155 (*Bgl*II-*Sca*I)		49,163
pExtet7	pEx31a (*Eco*RI)	1603 to 3673 (*Eco*RI-*Eco*RI)		93,366
pExtet15	pEx31c (*Eco*RI-*Hin*dII)	1810 to 3945 (*Hin*cII-*Hin*dIII)[i]		92,482

[a] Sites used for subcloning shown in brackets.
[b] Numbered according to Eisel et al. (8).
[c] Termination of transcription was obtained by cleavage downstream from the coding sequences. nt, Nucleotides.
[d] Deduced from the nucleotide sequence.
[e] The fragment was cloned into the *Bam*HI site of pUC12 and recovered by digestion with *Hin*dIII and *Sma*I.
[f] Ends were filled in with Klenow polymerase, and the fragment was subcloned into the *Sca*I site of M13E1 (26) and released with *Eco*RI and *Hin*dIII.
[g] Ends were filled in with Klenow polymerase.
[h] Protruding ends were removed with *E. coli* DNA polymerase.
[i] The fragment was cloned into pHK400 (8) and released with *Eco*RI and *Hin*dIII.

contained 15 μCi of L-[^{35}S]methionine and 2 μg of plasmid DNA.

Development of a cell-free protein-synthesizing system from *C. tetani* EK11. An S30 extract was prepared from *C. tetani* according to Pratt (27) with the following modifications. EK11 cells were grown to an optical density at 490 nm of 1.6. The cells were lysed under anaerobic conditions by a single passage through a French press. A low-molecular-weight mixture (LM-mix; 375 μl) was freshly prepared from stock solutions as follows: 40 μl of 2.2 M Tris-acetate, pH 8.2; 5 μl of 0.55 M dithiothreitol; 50 μl of 38 mM ATP, pH 7.0; 15 μl of a mix containing 88 mM each CTP, GTP, and UTP, pH 7.0; 100 μl of 0.42 M phosphoenolpyruvate, pH 7.0; 10 μl of amino acid mix (each amino acid at 55 mM, excluding methionine); 75 μl of 40% (wt/vol) polyethylene glycol 6000; 20 μl of folinic acid (2.7 mg/ml); 20 μl of *E. coli* tRNA (17.4 mg/ml); and 40 μl of salt solution (1.4 M ammonium acetate, 1.4 M potassium acetate, 0.38 M calcium acetate). The concentration of S30 extract was varied in a range between 10 and 60 μg of protein per assay mix, yielding optimal protein synthesis with 1 μl (20 μg of protein) of S30 extract. The time dependence of incorporation of radiolabeled methionine was followed for 15 to 75 min. Protein synthesis proceeded rapidly for 35 min and reached its highest levels after 45 min. The Mg^{2+} and K^+ concentrations were varied in the range of 5 to 25 mM and 0 to 120 mM, respectively. Optimal protein synthesis was obtained with 14 mM Mg^{2+} and 80 mM K^+. A 15-μl assay mix contained 1 μl of S30 extract, 3.75 μl of LM-mix, 13 mM magnesium acetate, 7.5 μCi of L-[^{35}S]methionine, and 2 to 5 μg of cesium chloride gradient-purified plasmid DNA. The mixture was preincubated without methionine and DNA for 20 min in 2.2-ml Eppendorf cups (wide bottom) in a rotating (270 rpm) water bath at 37°C. The reaction was started by the addition of [^{35}S]methionine and DNA. After a 40-min pulse, 40 μg of unlabeled methionine was added, and incubation was continued for 5 min.

Alternatively, the reactions were carried out in the presence of 10.0 μCi of [^{35}S]methionine diluted with unlabeled methionine to a final concentration of 5 μM. The samples were centrifuged for 4 min in an Eppendorf centrifuge and analyzed as described below.

Characterization of the expression products. Samples of the assay mixes were analyzed under reducing conditions by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 13% polyacrylamide gels. Immunoprecipitations with protein A-Sepharose were performed in RIPA buffer as described previously (26). For Western immunoblotting analyses, the method of Blake et al. (5) was used. Enzyme linked immunosorbent assay (ELISA) reactions were performed with the immunoglobulin G (IgG)-horseradish peroxidase method (33).

Immunization of rabbits with toxin-specific fusion proteins. Fusion proteins containing an N-terminal part of the MS2 polymerase fused in frame to the Tet5, Tet7, or Tet15 regions of the toxin were induced and purified as described before (8, 32). The enriched protein fraction (4.0 mg of protein per ml) was mixed with complete Freund adjuvant prior to immunization of 6-week-old New Zealand White rabbits. Each rabbit received about 100 μg of antigen, injected intradermally. Antibody titers were determined by ELISA with purified tetanus toxin as well as the corresponding fusion protein as antigens. For optimal titers, rabbits were boosted 3 weeks after the first injection with the same amount of antigen lacking adjuvant. Ten days later, serum was collected.

Inhibition of [^{3}H]norepinephrine release from rat brain homogenate. The experimental procedure has been published in detail previously (12). Briefly, prewashed rat brain homogenate was suspended at 2% (wt/vol) in Krebs-Ringer-HEPES buffer (KRH; 130 mM NaCl, 4.75 mM KCl, 2.54 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 11 mM glucose, 20 μM ascorbic acid, 10 μM isonicotinic acid 2-isopropylhydrazide) and incubated for 15 min at 37°C with the same volume of KRH containing 2 μCi of [^{3}H]norepinephrine per ml. After two washing steps with the same buffer lacking the radiolabeled compound, 200 μl of the

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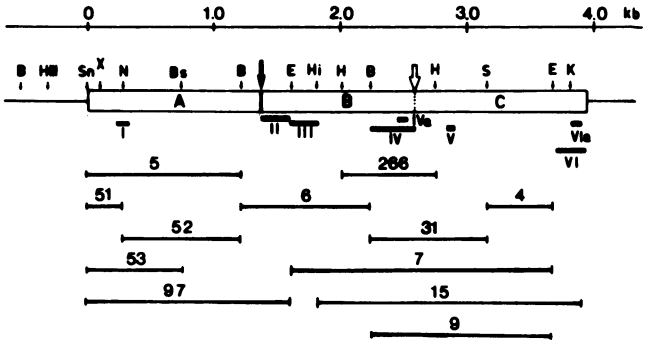

FIG. 1. Restriction map of the tetanus toxin gene. The open box corresponds to the coding region. Restriction sites used for cloning into expression vectors are indicated. Positions corresponding to the proteolytic cleavage sites between the A- and BC-fragments (solid arrow) and fragment-C (open arrow) are marked. Restriction sites used to map epitopes following in vitro transcription-translation or expression of individual gene fragments in *E. coli* are indicated: B, *Bgl*II; Bs, *Bsm*I; E, *Eco*RI; H, *Hpa*II; Hi, *Hin*cII; HIII, *Hin*dIII; K, *Kpn*I; N, *Nco*I; S, *Sca*I; Sn, *Sna*BI; X, *Xba*I. Solid bars indicate the locations of domains recognized by individual monoclonal antibodies as mapped in this study: I, 993D5 and 993D5B4; II, 161D6 and 162D2; III, 161A1; IV, 104A1; V, 164B4 and 165B2. Regions IVa, V, and VIa indicate positions of human T-cell-specific epitopes as mapped previously (7).

preloaded suspension (2% wt/vol) was mixed with 100 μl of a solution of tetanus toxin (200 ng/ml in KRH containing 0.1% bovine serum albumin) which had been preincubated for 30 min at room temperature with the individual antiserum or preimmune serum. After 2 h at 37°C, the samples were transferred to Millipore GF-C glass fiber filters and washed with KRH buffer for 15 min. For a 2-min pulse, the potassium ion concentration was increased to 31 mM, while the sodium ion concentration was simultaneously reduced to 106 mM. This treatment generally released about 40% of the total radiolabel from control samples. The capacity of individual antisera to abolish the inhibitory effects of tetanus toxin on release was determined by measuring the levels of extracellular and total radiolabeled norepinephrine. Each antiserum was tested in three independent experiments, yielding essentially identical results. Toxin neutralization studies involving monoclonal antibodies were initially performed with unpurified ascites fluids. When toxin neutralization was observed, tests were repeated with purified IgG at concentrations corresponding to the dilutions in Table 2.

RESULTS

In vitro synthesis of tetanus toxin-specific fragments from in vitro-synthesized mRNA. Our first approach to obtain defined toxin-specific peptides was based on the in vitro synthesis of mRNA and its subsequent translation in appropriate translation systems. Five different constructs containing different fragments of the toxin gene (Fig. 1) were prepared in pSP64 or pGEM vectors as explained in Table 1.

By using SP6 RNA polymerase and DNA templates that were linearized downstream from the toxin-specific sequences, mRNA of the expected size was obtained in microgram yields (data not shown). For subsequent translation assays in reticulocyte lysate, the mRNA was capped by adding the capping analog $m^7G5'ppp5'G$ in a 10-fold excess over GTP. In the wheat germ system, capping did not improve the translation efficiency, as also documented by others (29).

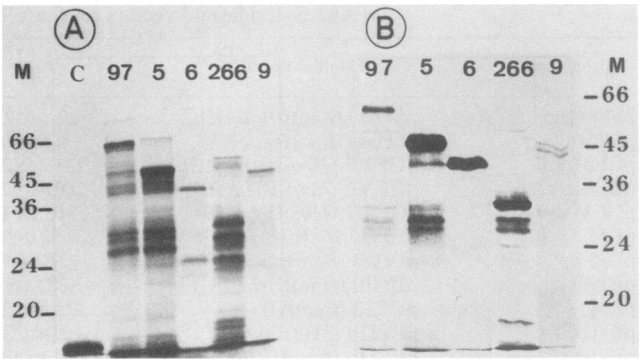

FIG. 2. In vitro translation of toxin-specific mRNA in wheat germ extract (A) or reticulocyte lysate (B). RNA encoding individual portions of tetanus toxin as indicated by the numbers above the lanes was obtained by in vitro transcription. The translation products were labeled with [^{35}S]methionine and analyzed on 13% polyacrylamide gels. Lane C, Control translation without exogenous RNA. M, Molecular masses of unlabeled marker proteins (in kilodaltons).

Figure 2 compares the results obtained by translating the individual mRNA species in wheat germ extract (A) and reticulocyte lysate (B). The peptides Tet97 and Tet5 had the authentic N terminus of the toxin molecule; Tet97, however, contained the entire Tet5 sequence and 131 additional C-terminal amino acids. Although in both translation systems peptides of the size corresponding to the coding capacity (Table 1) were obtained, smaller peptides of identical size were formed from *tet97* and *tet5* mRNAs, indicating that these smaller products could arise from premature termination of translation. The formation of the second-largest product obtained from pGEMtet9 could result from ribosomal slippage and initiation at a second AUG codon 61 nucleotides downstream from initiation codon 2427. In general, the reticulocyte lysate system yielded larger amounts of peptides than were obtained in the wheat germ system. It should be noted, however, that both eucaryotic systems were less efficient in translating internal or C-terminal sequences of the toxin molecule.

Cell-free protein synthesis in *E. coli* or *C. tetani* lysates. The constructs pEJ5 and pEJ97 contained 5′ noncoding sequences harboring the toxin promoter shown to be functional in *E. coli* (8). Figure 3 shows that polypeptides of the expected size, 64 and 47 kilodaltons (kDa), were obtained. The replacement of the toxin promoter by strong *E. coli* promoters (such as *lac* or *tac*), as well as the replacement of the clostridial Shine-Dalgarno sequence by a synthetic sequence corresponding to the *ompA* gene, did not improve yields. In addition, neither modification of ionic conditions nor alteration of the spermidine or ATP concentration led to full-sized products (data not shown). In some instances, as shown for pEJ6 (Fig. 3, lane 6), the majority of radiolabel was apparently incorporated into β-lactamase (indicated by the arrowheads). The recombinant plasmid should yield a toxin-specific 41,583-Da protein corresponding to the largest molecular species barely detected in lane 6. For comparison, see the peptides synthesized from pAT153 control DNA, also carrying the ampicillin resistance gene, in lane +c. Linearization of the plasmids rather diminished the yields of peptides.

For these reasons, we developed a cell-free protein-synthesizing system from a nontoxigenic strain of *C. tetani*. Under optimized conditions, a 30-fold stimulation of protein

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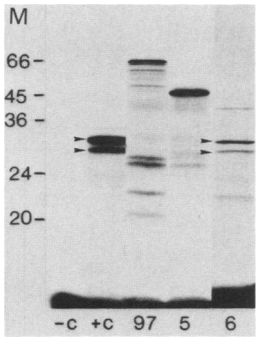

FIG. 3. Cell-free protein synthesis in the *E. coli* system. [^{35}S]methionine-labeled products were analyzed by SDS-PAGE as before. Supercoiled DNA, obtained by centrifugation on cesium chloride gradients, was used from various recombinants. Lane −C, No added DNA; lane +C, pAT153; lanes 97, 5, and 6, products obtained from pEJ97, pEJ5, and pEJ6, respectively. M, Molecular mass markers (in kilodaltons).

synthesis was obtained with 10 μg of exogenous total RNA from *C. tetani* (Fig. 4A). It should be noted that a plateau value was not reached even at the highest RNA concentration tested (666 μg/ml). Stimulation by exogenous DNA was less efficient than stimulation by RNA. In combined transcription-translation assays, the best results were obtained with 2 to 5 μg of supercoiled DNA, yielding a 3.5-fold stimulation of protein synthesis.

The clostridial system was superior to the previous systems since it yielded predominantly full-sized peptides, irrespective of whether the toxin-specific sequences were derived from the N- or C-terminal part of the molecule (Fig. 4B). With none of the other systems could the 23,100-Da peptide, representing Tet4 (Fig. 1), be obtained in adequate amounts. This peptide was expressed at high levels under control of the toxin promoter (Fig. 4B, lane d). The clostridial system, however, was not very efficient when expression was driven by the *lac* promoter. From pEJ6, for example, the largest detectable peptide (32,000 Da) was considerably smaller than the expected 41,580-Da species. In vitro studies involving RNA polymerase purified from *C. tetani* indicated that this polymerase transcribed genes under control of the *lac* or *tac* promoter, albeit at a reduced efficiency (U. Eisel and H. Niemann, unpublished).

Mapping of antigenic determinants. In a first attempt to apply peptides synthesized in the clostridial system in the characterization of 22 different monoclonal antibodies raised against tetanus toxin or toxoid (1), we performed immunoprecipitation reactions (Table 2). Figure 4C shows an example of such an experiment. Antibody 993D5 precipitated the Tet97 and Tet5 peptides equally well (lanes a and c), while antibody 161D6 or 162D2 recognized Tet97 exclusively, indicating that the corresponding epitope was located within the additional C-terminal sequences present in Tet97. Since these latter two antibodies were specific for the BC-fragment (Table 2), their epitopes had to reside within region II in Fig. 1.

To our surprise, however, only eight monoclonal antibodies (Table 2) could be applied successfully in immunoprecipitations of in vitro-translated peptides. The amounts of recombinant peptides obtained by in vitro translations were not sufficient to be analyzed by ELISA or Western blotting. Therefore, we extended our studies to fusion proteins synthesized in *E. coli* (Fig. 5). Monoclonal antibody 993D5

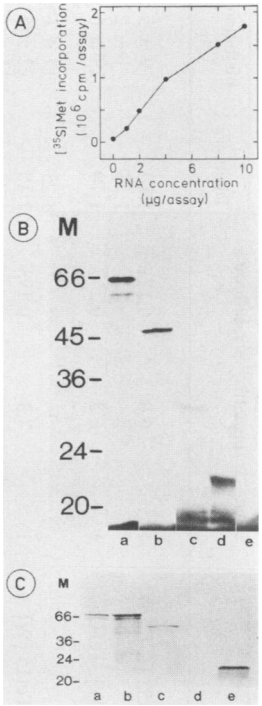

FIG. 4. Cell-free protein synthesis in a system from *C. tetani*. (A) Stimulation by exogenous RNA. Total RNA was isolated from *C. tetani* E88 by phenol-guanidinium hydrochloride and added to a 15-μl translation assay mix containing 1 μl of S30 extract, 14 mM Mg^{2+}, 80 mM K^+, and 10 μCi of [^{35}S]methionine. After incubation for 45 min at 37°C, radiolabel incorporation was determined by trichloroacetic acid precipitation in two independent sets of experiments. (B) Combined transcription-translation. Supercoiled DNA (2 μg) of various recombinants was transcribed and translated in the cell-free system as detailed in Materials and Methods. [^{35}S]methionine-labeled samples were analyzed by SDS-PAGE and autoradiography. Lane a, Peptides obtained from pEJ97; lane b, peptides from pEJ5; lane c, peptides from pEJ6; lane d, peptides from pEJ4; lane e, no DNA added. M, Molecular mass markers (in kilodaltons). (C) Immunoprecipitation of individual peptides with monoclonal antibodies. Lanes a and b, Peptides obtained from pEJ97 precipitated with monoclonal antibody 161D6 or 993D5, respectively; lane c, peptides obtained from pEJ5 immunoprecipitated with L-chain-specific monoclonal antibody 993D5; lane d, the same as in lane c except that antibody 161D6 was used; lane e, immunoprecipitation of the products obtained from pEJ4 with the anti-Tet15 polyclonal rabbit serum.

bound to fusion proteins containing Tet5 and Tet97 sequences as well as those containing Tet53. In contrast, fusion proteins consisting of Tet52 or Tet51 were not recognized, although such fusion proteins were equally well precipitated with a polyclonal serum. These findings suggest that both antibodies recognized an epitope (region I in Fig. 1) located in the vicinity of amino acid 90, the border between the Tet51 and Tet52 sequences. Computer-assisted analyses of the toxin sequence by the method of Hopp and Woods (14) indicated a strong epitope, D(81)SDKDR(86), in this domain. This predicted epitope had the highest A_h value (2.55) of the entire toxin molecule.

The data obtained with the MS2-Tet fusion proteins confirmed the results obtained by immunoprecipitation of in vitro-translated peptides (Table 2). No additional epitopes could be detected with this experimental approach, however.

Inhibition of the effects of tetanus toxin on rat brain

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TABLE 2. Specificity and toxin-neutralizing properties of individual antibodies

Antibody	Specificity[a]		IgG class	Concn (mg/ml)[b]	ELISA reactivity[c]	Inhibition of toxin effects[d]	
	Reactive	Nonreactive				Dilution	Inhibition (%)
Monoclonal							
993D5	A [I], Tet5 [W], Tet53 [W], Tet97 [I, W], region I	BC [I, E, W], Tet51 [I, E, W], Tet52 [I, E, W]	1	6.0	0.171	1:10	9 ± 5
						1:25	1 ± 2
161D6	BC [I, E, W], Tet6 [I, W], Tet97 [I, W], region II	A [I, E, W], Tet5 [I, W], Tet7 [I, W], Tet15 [I, W]	1	6.2	0.177	1:10	19 ± 6
						1:25	17 ± 6
162D6	BC [I, E, W], Tet6 [W], Tet97 [W], region II	A [I, E, W], Tet5 [W], Tet7 [W], Tet15 [W]	1	7.3	0.193	1:10	22 ± 5
						1:25	16 ± 6
161A1	BC [E, W], Tet7 [E, W], region III	A [I, E, W], Tet15 [W]	1	5.0	0.137	1:10	0
						1:25	0
104A1	BC [I, E, W], Tet266 [I, W], Tet7 [E, W], Tet15 [E, W], Tet31 [E, W], region IV	A [I, E, W], C [I, E, W]	1	0.8	0.067	1:10	61 ± 9
						1:25	55 ± 6
164B4	BC [E, W], C [E, W], Tet31 [E, W], Tet7 [E, W], Tet15 [E, W], 947-FNNFTVSFWLRVPKVSA SHLE-967, region V	A [I, E, W], Tet4 [E, W], Tet266 [E, W]	1/2B	7.0	0.092	1:10	0
						1:25	0
165B2	BC [I, E, W], C [E, W], Tet31 [E, W], 947-FNN FTVSFWLRVPKVSASHLE-967, region V	A [I, E, W], Tet4 [E, W], Tet266 [E, W]	1/2B	8.2	0.113	1:10	0
75A4	BC [I, E, W], C [I, E, W], Tet15 [I, E, W], region VI	Tet6 [I, E, W], Tet7 [I, E, W]	2	6.5	0.172	1:100	100 ± 2
						1:1,000	100 ± 2
						1:10,000	79 ± 6
Control ascites			1	8.0	0.008	Undil	0
Rabbit antisera							
Anti-Tet5	Tet5 [I, E, W]				0.239	Undil	12 ± 5
						1:10	6 ± 5
						1:25	0
Anti-Tet7	Tet7 [I, E, W]				0.208	Undil	100 ± 3
						1:10	67 ± 8
						1:25	36 ± 9
Anti-Tet15	Tet15 [I, E, W]				0.241	Undil	100 ± 10
						1:10	82 ± 8
						1:25	74 ± 9

[a] The reactivity of antibodies to specific subfragments was determined by immunoprecipitation [I], Western blotting [W] (5), or ELISA [E] (33). For locations of the fragments see Fig. 1.
[b] Determined by affinity purification and ELISA with subclass-specific antibodies.
[c] Determined at an antibody dilution of $1:10^4$ by ELISA with 10 μg of purified tetanus toxin per ml.
[d] Expressed as the mean of triplicate values determined by the norepinephrine release assay in vitro.

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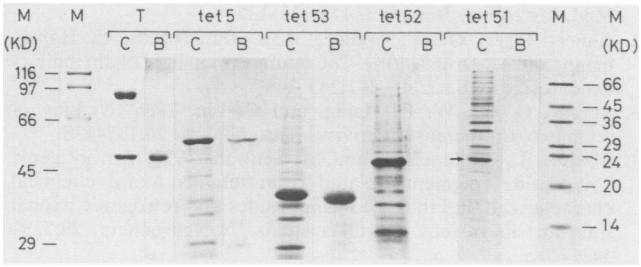

FIG. 5. Mapping of the epitope for monoclonal antibody 993D5 with MS2-toxin fusion proteins. Specific regions of the toxin gene as indicated were cloned in frame into pEx31 vectors and transfected into *E. coli ts*537 cells. Cultures were grown overnight at 32°C (nonpermissive temperature). Synthesis of the fusion proteins was induced by diluting the cultures fivefold in medium preheated to 42°C (permissive temperature). Incubation was continued for 4 h. Cells were lysed and extracted as described by Strebel et al. (32), separated by SDS-PAGE, and analyzed by Western blotting with monoclonal antibody 993D5. Note that the products from tet51 were analyzed on a 15% gel together with the molecular mass markers (M) on the right-hand side. The arrow indicates a band not present in control cells or cells expressing other fusion proteins. The corresponding material was immunoprecipitable with polyclonal rabbit sera against Tet5 (not shown). T, Tetanus toxin; C, gels were stained with Coomassie brilliant blue; B, after blotting onto nitrocellulose sheets and incubation with the monoclonal antibody at 60 μg of protein per ml. Bound antibody was visualized with peroxidase-conjugated goat anti-mouse IgG as previously described (5).

homogenate. The toxin-neutralizing properties of the monoclonal antibodies listed in Table 2 were tested together with those of polyclonal sera raised against fusion proteins in an in vitro assay based on the inhibitory effects of tetanus toxin on the release of [^{3}H]norepinephrine from brain homogenate prelabeled with the hormone (12). The results are summarized in Table 2 and Fig. 6. Two of the monoclonal sera, 104A1 and 75A4, were capable of completely blocking the toxin effect. Similar results were obtained with polyclonal rabbit sera directed against parts of the BC-fragment. In contrast, antibodies that recognized domains within the A-fragment, such as 993D5 and anti-tet5, were less efficient in neutralizing the toxin in this in vitro assay.

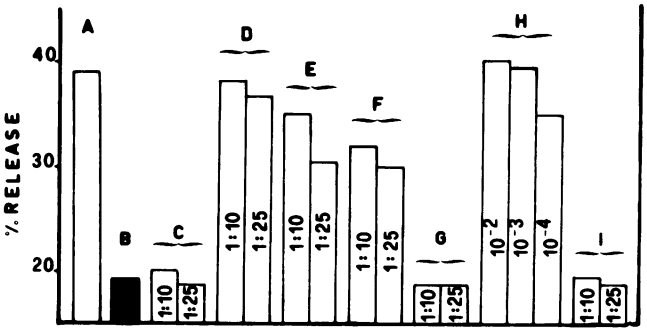

FIG. 6. Toxin-neutralizing properties of polyclonal and monoclonal antibodies. Rat brain homogenate was preloaded with [^{3}H]norepinephrine and treated for 2 h with tetanus toxin (67 ng/ml, final concentration) or toxin that was preincubated with individual antisera at the dilutions indicated. After a 2-min pulse with 31 mM K^+, the levels of extracellular and cellular norepinephrine were determined by scintillation counting. The results are expressed as the mean of triplicate determinations in independent experiments. Fur further details, see Materials and Methods. (A) No tetanus toxin added. (B) Tetanus toxin without antisera. (C through I) Tetanus toxin was preincubated with one of the following antisera: C, anti-Tet5; D, anti-Tet15; E, anti-Tet7; F, monoclonal 104A1; G, monoclonal 164B4; H, monoclonal 75A4; I, negative control ascites. For IgG concentrations of monoclonal antibodies, see Table 2.

DISCUSSION

We used recombinant tetanus toxin-specific peptides to characterize the epitopes of monoclonal antibodies whose toxin-neutralizing activity was determined in an in vitro assay. Four different cell-free systems were compared for their efficiency in producing subfragments of the toxin molecule in vitro from cloned DNA. This study was carried out because previous experiments had indicated that clostridial DNA with a G+C content of merely 27% was expressed at low levels in *E. coli* (8). Our studies showed that clostridial DNA yielded preferentially full-sized peptides in a homologous cell-free system developed from a nontoxigenic strain of *C. tetani*. Optimization studies revealed a similar dependency of this system on Mg^{2+} (13 to 15 mM) and K^+ (80 mM) as described for systems from *E. coli* (27), *Bacillus subtilis* (17, 20, 36), and *Clostridium pasteurianum* (30, 31). Total RNA from *C. tetani* stimulated protein synthesis in the clostridial system about 30-fold at a concentration of 300 μg/ml. Stallcup et al. (31) reported a sixfold stimulation with similar amounts of RNA with a reconstituted S30 extract from *C. pasteurianum*. Only a 3.5-fold stimulation, however, was obtained with supercoiled exogenous DNA containing the promoter of the toxin gene. Fractionation and reconstitution of the S30 extract according to protocols published for *C. pasteurianum* (31) did not improve the levels of transcription in our system.

In reticulocyte lysate and wheat germ extract systems, the translation efficiency of presynthesized toxin-specific mRNA was quite low in comparison to that of standard globin or coronaviral mRNA (19). In both systems, premature termination of translation was a major reason for the reduced yields. The combined transcription-translation system for *E. coli* did not prove satisfactory with clostridial DNA. Even though ribosomes from gram-negative bacteria have been reported to be less restricted with respect to the nature of the mRNA than ribosomes from gram-positive organisms (31), exogenously added total clostridial RNA was translated quite inefficiently in the *E. coli* system (not shown). In addition, peptides encoded by entirely toxin-specific DNA were again poorly synthesized in vivo, while MS2 fusion proteins were made in normal amounts, as shown in Fig. 5. These findings suggest that in this system, initiation of translation could play the crucial role.

Using such recombinant peptides, we coarsely mapped the epitopes of eight monoclonal antibodies. Of a total of 22 different antisera, all of which were shown previously to bind to tetanus toxin (1), only 8 reacted with toxin fragments produced either in vitro or in vivo, as determined by immunoprecipitation, ELISA, or Western blotting. These eight monoclonal antibodies recognized six different epitopes.

None of the eight antibodies alone was capable of neutralizing 10 50% lethal doses when coinjected into mice; each only prolonged the time to death. Similar observations have been reported previously (24, 34). However, such in vivo experiments are costly in terms of test animals and monoclonal antisera. Therefore, to assess the toxin-neutralizing activity of monoclonal antibodies, we used an in vitro system in which the essential steps of the toxification process, namely, binding and internalization of the toxin, are maintained. Our data demonstrate that sera directed against the A-subunit of tetanus toxin, such as polyclonal rabbit

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anti-Tet5 or the monoclonal antibody 993D5, which could be used in immunoprecipitations and thus presumably also bind to the toxin in solution, barely neutralized the toxin. Similar properties of monoclonal antibodies against the A-fragment have been reported previously (34). Three epitopes were mapped with the help of monoclonal antibodies in the B-fragment. This domain of the toxin has been suggested to play an important role in the interaction of the toxin with a putative proteinaceous receptor (25) and could be involved in the translocation of the A-subunit into the cytosole (reviewed in reference 13). One of the antibodies (104A1) caused marked neutralization of the toxin by binding to region IV, while antibodies 162D2 and 161D6, both recognizing region II of the toxin and both useful in immunoprecipitations, only reduced the toxin effects to about 20% of the value without antibody. Unfortunately, no conclusions concerning binding of antibodies to epitopes III and V can be drawn, since the corresponding antibodies (161A1 for region III, 164B4 and 165B2 for region V) did not bind to radiolabeled tetanus toxin in solution (data not shown). In competitive ELISA reactions, binding of the latter two antibodies to region V could be specifically inhibited by the synthetic peptide NH_2-F(947)NNFTVSFWLRVPKVSASHLE(967), carrying a human T-cell epitope (7). Apparently region V becomes exposed at the periphery of the molecule only after treatment with detergent or, in the host, after proteolytic processing by antigen-presenting cells. Our finding suggests that B cells and T cells can principally recognize the same epitope. We cannot exclude, however, that a peptide of 21 amino acids exhibits more than the one epitope and that the murine B-cell and the human T-cell clones therefore recognize two different sites. Recently, a pentapeptide sequence from human cytomegalovirus was demonstrated to be sufficient for major histocompatibility complex-restricted T-cell stimulation (28). Systematic modifications of the peptide from either end will now allow the precise characterization of the T- and B-cell epitope(s). In agreement with published observations (10, 15, 34), antibodies binding to fragment-C showed the most pronounced toxin-neutralizing activities. This is not surprising, since fragment-C has been shown to be nearly as effective as the holotoxin with respect to binding to thyroid or neural membranes or to certain gangliosides. Recent studies have indicated that monoclonal antibody 75A4 indeed inhibits binding of tetanus toxin to gangliosides (H. Niemann, unpublished observation). Epitope VI is localized within the 80 carboxy-terminal amino acids. Although steric hindrance caused by the bound antibody cannot be excluded as an indirect reason for inhibition of toxin binding, it is possible that this part of the toxin molecule participates directly in the binding to the ganglioside receptor.

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